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SENSITIZATION OF GUINEA PIGS TO CHROMIUM SALTS*

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Allergic contact dermatitis to chromates has been recognized for many years. Recently cross-reactions between hexavalent and trivalent chromium salts have been reported. Several questions remain unanswered. Why is hexavalent chromium more immunologically active? Do percutaneous absorption rates account for all the clinical differences observed? What is the importance of the carrier-protein? Recently, we have sought these answers employing guinea pigs as an experimental model.

As a preliminary step in the study of reactions of guinea pigs to chromium-protein complexes, attempts were made to sensitize the animals to potassium dichromate and chromic chloride. When the techniques of Hunziker (1) and Van Neer (2) were employed, the results were inconsistent and disappointing. The rate of successful sensitization, as determined by delayed papular reactions to intradermal tests, never exceeded 20%. Therefore, we reevaluated and modified the procedures, until after several trials our techniques gave consistently successful results. The details are presented along with other pertinent information regarding cross-reactivity of several chromium salts.

PROCEDURE

Albino guinea pigs weighing 300 and 500 Gm. were sensitized to hexavalent chromium ($K_2Cr_2O_7$) by three subcutaneous injections in the nape one week apart. The following emulsion was injected: 0.5 cc Freund's complete adjuvant (Difco) with 0.5 cc of 3.4×10^{-3} M of $K_2Cr_2O_7$.‡ Three weeks later the animals were tested with intradermal injections in clipped or epilated skin. The eliciting dose was 0.1 cc of 4.2×10^{-4} M $K_2Cr_2O_7$ in physiologic saline. This produced, at 48 hours, a well-defined indurated erythematous papule, at least 10 mm in diameter. In control animals no reaction was present at 48 hours. Weaker reactions were usually obtained with test concentrations of 2.1×10^{-4} M; below this level results were inconsistent.

With the same procedure, guinea pigs were sensitized to chromic chloride; the sensitizing injections contained 0.5 cc of Freund's complete adjuvant with 0.5 cc of 3.4×10^{-2} M $CrCl_3$. The test dose was 0.1 cc of 4.2×10^{-4} M $CrCl_3$ in physiologic saline intradermally. This produced no reaction whatsoever in thirty control animals.

Having established delayed hypersensitivity to hexavalent and trivalent chromium compounds, several sets of experiments relative to cross-reactions were performed. Animals sensitized with $K_2Cr_2O_7$ or $CrCl_3$ were tested with both compounds and a number of additional trivalent salts. Moreover, they were also exposed to conjugates of chromium linked with plasma proteins and skin extract proteins (modification of the technique of Salvin and Smith) (4, 5).

RESULTS

Initial sensitization: After the above sensitization to $K_2Cr_2O_7$, twenty-six of twenty-seven previously unexposed guinea pigs developed positive skin tests to $K_2Cr_2O_7$. In all cases the 48 hour intradermal skin test result was an indurated erythematous papule of at least 10 mm (+1 response). In twenty-three of the animals the response measured over 15 mm (+2); in twelve the response was greater than 20 mm (+3); and in two, central necrosis developed in the reaction (+4).

When $CrCl_3$ was used for sensitization, ten of

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‡ A concentration 3.4×10^{-3} M is equivalent to 0.1% solution (W/V) of $K_2Cr_2O_7$, each 0.5 cc containing 0.5 mg of the salt; so that each guinea pig receives a total of 1.5 mg of $K_2Cr_2O_7$. This represents the reference concentration for serial dilutions. We are in complete agreement with Epstein (3), who has stressed the importance of expressing concentrations in molarity rather than weight/volume as this usage allows for immediate, accurate comparison of the actual quantity of the Cr ion in solutions of different Cr salts, e.g.:

Molarity	$K_2Cr_2O_7$ (W/V)	$CrCl_3 \cdot 6H_2O$	$Cr_2(SO_4)_3 \cdot 15H_2O$	$Cr(NO_3)_3 \cdot 9H_2O$
3.4×10^{-3}	0.10%	0.090%	0.19%	0.14%
8.5×10^{-4}	0.025%	0.022%	0.048%	0.034%
4.2×10^{-4}	0.012%	0.011%	0.024%	0.017%
2.1×10^{-4}	0.006%	0.006%	0.012%	0.009%

thirteen animals developed positive reactions (at least +); of these, four were read as (+2). None developed (+3) or (+4) reactions.

Cross-reactions: Of the twenty-six guinea pigs sensitized to $K_2Cr_2O_7$ (Cr_{VI}), all reacted to $CrCl_3$ (Cr_{III}) (see Table I). In most cases the reaction to Cr_{VI} was of greater magnitude, but in seven animals the degree of reactivity was the same and in one there was a greater reaction to Cr_{III} than to Cr_{VI}. The difference in reactivity to Cr_{VI} and Cr_{III} is significant at P= 0.005, employing the Wilcoxon matched pairs, signed-rank test (6).

TABLE I

Skin test reactions to potassium dichromate and chromic chloride in guinea pigs sensitized to potassium dichromate (see text for techniques)

Guinea pig no.	Reaction to potassium dichromate	Reaction to chromic chloride
	*	
1	+2	+2
2	+1	+1
3	+2	+1
4	+1	+1
5	+2	+2
6	+2	+2
7	+2	+2
8	+2	+1
9	+2	+1
10	+3	+2
11	+3	+2
12	+3	+2
13	+1	+2
14	+3	+2
15	+4	+2
16	+3	+2
17	+3	+2
18	+2	+1
19	+3	+1
20	+2	+2
21	+3	+1
22	+2	+2
23	+3	+2
24	+3	+1
25	+2	+1
26	+3	+2

* Note: (−) = no response; (±) = equivocal response, less than 10 mm diameter; (+1) = response of 10 mm diameter; (+2) = response of 15 mm diameter; (+3) = response of 20 mm diameter; (+4) = response of 20 mm diameter with central necrosis.

TABLE II

Skin test reactions to potassium dichromate and chromic chloride in guinea pigs sensitized to chromic chloride

Guinea pig no.	Chromic chloride	Potassium dichromate
1	+1	+1
2	+2	+1
3	+1	+2
4	+1	+1
5	+1	+1
6	+2	+2
7	+1	−
8	+2	+1
9	+1	+1
10	+2	−

TABLE III

Skin test reactions to chromium salts in guinea pigs which had been sensitized to potassium dichromate (see text for concentrations employed)

Guinea pig no.	Potassium dichromate	Chromic acetate	Chromic chloride	Chromic nitrate	Chromic sulfate	Chromic oxalate
1	+3	+2	+2	+3	+2	±
2	+3	+2	+2	+3	+3	±
3	+2	+2	+2	+2	+2	−

Of the ten guinea pigs sensitized to $CrCl_3$, eight also reacted to $K_2Cr_2O_7$ (see Table II). In five cases the degree of reactivity was identical. In one case there was a greater reaction to Cr_{VI} than Cr_{III}. The differences in reactivity are not statistically significant.

Reactions to other trivalent salts: Studies of a similar nature were conducted with four other solutions of trivalent chromium salts. The highest concentrations which were not irritating to control animals were determined by serial dilution and subsequently used for skin testing. They are as follows: Chromic acetate (2.5×10^{-3} M), chromic nitrate (9.6×10^{-4}), chromic oxalate (2.5×10^{-4} M), chromic sulfate (2.4×10^{-4} M). Three guinea pigs highly sensitive to $K_2Cr_2O_7$ had significant cross-reactions to the acetate, nitrate, chloride, and sulfate, but not the oxalate salt (see Table III). Three animals sensitized to $CrCl_3$ showed weaker cross-reactions to the same compounds (see Table IV).

Attempts to sensitize guinea pigs with the

TABLE IV

Skin test reactions to chromium salts in guinea pigs which had been sensitized to chromic chloride

Guinea pig no.	Chromic chloride	Potassium dichromate	Chromic acetate	Chromic nitrate	Chromic sulfate	Chromic oxalate
1	+1	+1	−	+1	+1	±
2	+2	+1	+1	+1	+1	±
3	+2	−	+1	+2	+1	−

above trivalent salts, other than $CrCl_3$, were uniformly unsuccessful. Concentrations of 1.7×10^{-2} M to 5×10^{-2} M were used with Freund's complete adjuvant in a schedule identical to that which had been successfully used for $K_2Cr_2O_7$ and $CrCl_3$.

Reactions to chromium-protein conjugates: It was not possible to sensitize guinea pigs to conjugates of $K_2Cr_2O_7$ with guinea pig serum albumin, globulin, or guinea pig skin extract, by using Freund's adjuvant and the above described schedule. The experiments with $CrCl_3$ conjugated to the same proteins also failed to evoke sensitivity to either the conjugate or the salt by itself. Similarly, when animals which were sensitized to either $K_2Cr_2O_7$ or $CrCl_3$ with Freund's complete adjuvant, as described above, were tested with chromium-protein conjugates (VI or III), they failed to react, even though they showed definite reactions to the simple unconjugated salts. It should be emphasized that the chromium content of these conjugates, as determined by tracer technique with labeled Cr^{51}, was in the same range as that of the simple solutions to which the animals were highly reactive (2.1 to 5.0×10^{-4} M).

DISCUSSION

The techniques described above for inducing sensitization to chromium are reliable and relatively simple. Though Hunziker's method (1) is similar, these modifications have greatly increased the success rate employing dichromate and have, for the first time, allowed consistent achievement of sensitivity in animals using a trivalent salt. The sensitization is long lasting. Animals which were followed for one year retained their reactivity. Frequently the animals developed ulceration at the site of injection of the sensitizing emulsion but this invariably healed in two to three weeks. This occurred with both $K_2Cr_2O_7$ and $CrCl_3$, unlike the typical "chrome ulcers" seen clinically and experimentally which are produced only by hexavalent chromium (7). Control animals injected only with Freund's complete adjuvant did not develop local ulceration. The mechanism of this reaction at the injection site probably represents local irritation. Successful sensitization occurred in many animals which did not develop ulceration.

Cross-reactivity between hexavalent and trivalent chromium salts has been demonstrated in sensitized subjects (8, 9, 10). This has been confirmed in guinea pigs by our present experiments. Yet there can be little doubt that $K_2Cr_2O_7$ is a more effective sensitizer, even when differences in percutaneous absorption are obviated by use of intradermal injections. This has also been the conclusion of Mali *et al.* (11). The reactions to the hexavalent salt were consistently greater in degree. Sensitization was achieved with concentrations ten fold less than $CrCl_3$. Moreover, animals sensitized to $K_2Cr_2O_7$ showed more cross-reactions, which may be interpreted as an indication of a higher degree of sensitivity. Similarly, Fregert and Rorsman (12) have shown that the hexavalent chromium salts are more effective elicitors after sensitization has been established.

There are significant variations in the sensitizing and eliciting capacities of the several trivalent salts tested. The more highly dissociated salts produced more reactions than weakly dissociated salts. We have previously postulated that differences in epicutaneous sensitivity were due to differences in diffusion through the epidermis and have confirmed this by diffusion rate measurements *in vitro* (13). However, since this route was by-passed in these studies, we are led to the conclusion that the differences in reactivity are also in part inherent in the specific chromium salts. They are probably a function of their degree of dissociation, though Fregert and Rorsman (12) believe solubility in the physiologic range of pH is the important factor. The chromium ion itself undoubtedly is the hapten responsible for sensitization. The clinical and experimental differences observed among various chromium salts reflect the degree to which the chromium ion is available (through absorption and dissociation) to form a complete antigen by conjugation with a suitable carrier protein. Experiments quantitating the degree of binding to

protein strongly suggest that the ion is in the trivalent state when conjugation occurs (5). Hexavalent chromium is reduced *in vitro* before it is bound. It is possible that the redox reaction with Cr_{VI} provides a protein carrier with stronger affinity for binding or that the trivalent chromium as might develop from the interaction is predisposed to form a hapten-protein complex with a linkage of greater relevancy or the same hapten may become attached to two different points on the same protein serving to define two different antigenic determinations.

Benacerraf and Gell (14) and Gell and Silverstein (15) have masterfully demonstrated that in delayed allergy to simple chemicals there is specificity to the carrier protein as well as the hapten. For example, guinea pigs sensitized to conjugates of picryl and albumin are more reactive to that conjugate than they are to a conjugate of picryl and globulin. We had hoped this principle would apply to our work with conjugates of chromium and various proteins and could determine what protein binds with chromium *in vivo* to form the complete antigen. Unfortunately, this experiment was unsuccessful. However, since *in vitro* studies (5) have indicated that the chromium is bound to free amino and free carboxyl groups on the protein molecule, we are currently extending this work, using conjugates of chromium to the synthetic polypeptides poly-l-lysine and poly-l-glutamic acid, which should provide more information on carrier specificity.

SUMMARY

Techniques are outlined for the consistent production of delayed hypersensitivity to chromium in guinea pigs. Cross-reactions among various hexavalent and trivalent salts are readily elicited when differences in percutaneous absorption are obviated. A theory of sensitization to chromium is presented which we hope will clarify the controversy over valency and explain the differences observed clinically and experimentally.

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